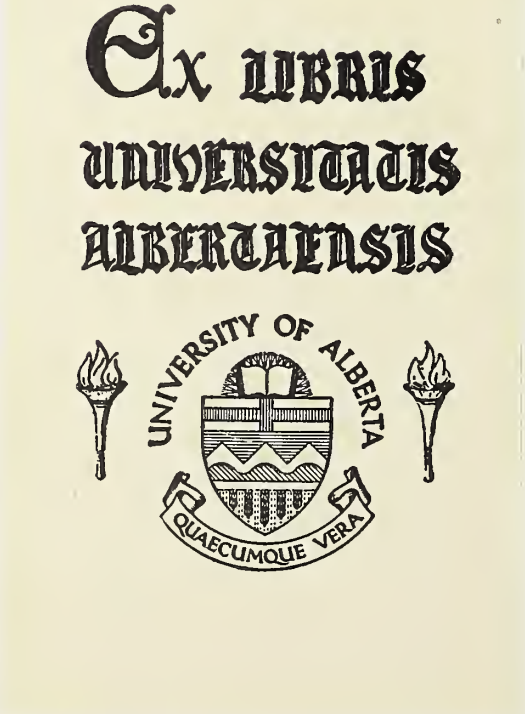

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THE METABOLISM OF PURINE RIBONUCLEOSIDES BY EHRLICH ASCITES TUMOR CELLS *IN VITRO*

by

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A THESIS

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ABSTRACT

The metabolism of adenosine and guanosine by cells of the Ehrlich ascites carcinoma *in vitro* was investigated. These cells cleaved inosine at rates which exceeded those for adenosine deamination by a factor of at least three; yet during the metabolism of adenosine *in vitro*, inosine appeared in the incubation medium. Only a small part of this anomalous appearance of extracellular inosine could be attributed to extracellular adenosine deaminase released into the medium by the tumor cells. A cell surface location for adenosine deaminase also did not explain this result because adenosine deaminase is a soluble enzyme and, furthermore, time studies showed that during the metabolism of adenosine-8-^{14}C by these cells an intracellular concentration of inosine was achieved which was several times larger than that of the medium. These data suggested that an intracellular compartmentation of adenosine deamination might be responsible for appearance of extracellular inosine.

Guanosine was metabolized by tumor cells to guanine, xanthine, uric acid and nucleotides, but was not deaminated to xanthosine. In addition, these cells synthesized guanosine from guanine in the presence of particular "ribosyl" donors. The latter reaction was enhanced by arsenate, iodoacetate, and 2,4-dinitrophenol.

6-(Methylmercapto)purine ribonucleoside markedly inhibited the metabolism of guanosine, in agreement with its effect on the metabolism of several other ribonucleosides; however, the metabolism of adenosine appeared to be less affected by the analogue. In all cases, cell integrity was required for inhibitory action. These findings have some implications toward the mechanism of ribonucleoside transport.

ACKNOWLEDGEMENT

I wish to express sincerely my deep gratitude to my supervisor, Dr. A.R.P. Paterson, for his enthusiastic guidance and invaluable advice during these studies. I am also grateful to the members of McEachern Laboratory who have given me help and advice.

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LIST OF ABBREVIATIONS

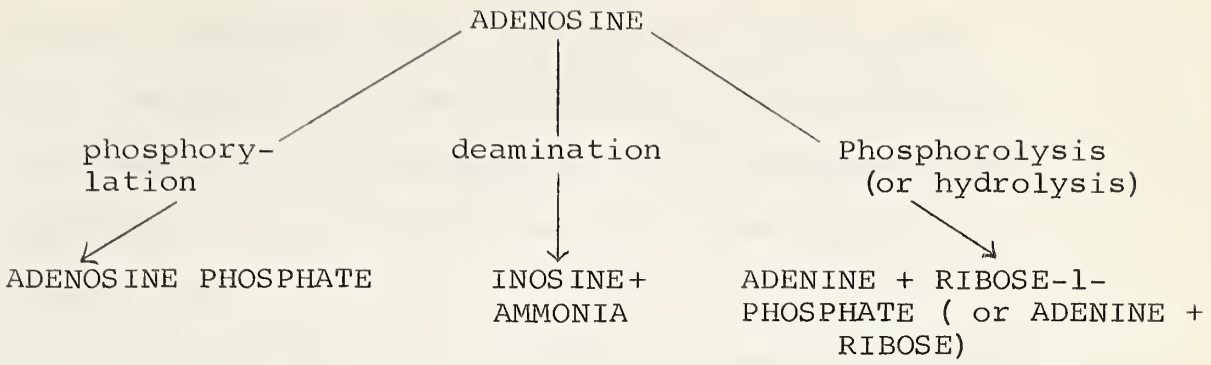
H	hypoxanthine
G	guanine
X	xanthine
Ur	uric acid
6MP	6-mercaptopurine
AR	adenosine
HR	inosine
GR	guanosine
6MPR	6-mercaptopurine ribonucleoside
Me-6MPR	6-(methylmercapto)purine ribonucleoside
AMP	adenosine 5'-monophosphate
GMP	guanosine 5'-monophosphate
GDP	guanosine 5'-diphosphate
GTP	guanosine 5'-triphosphate
PRPP	5-phosphoribosyl pyrophosphate
c.p.m.	counts per minute
PCA	perchloric acid
Tris	tris-(hydroxmethyl)aminomethane
Versene	disodium ethylene diamine tetraacetate
POPOP	1,4-di(2-(5-phenyloxazolyl))-benzene
PPO	2,5-diphenyloxazole

I. INTRODUCTION

This investigation is a part of a more general inquiry into the metabolism of ribonucleosides by Ehrlich ascites tumor cells. Studies of ribonucleoside metabolism in animal tissues have been conducted mainly with tissue extracts and have usually been concerned with phosphorolysis. The present studies are a departure from this trend in that they concern ribonucleoside metabolism in intact cells. During studies in this laboratory of the metabolism of certain purine analogue nucleosides, it became apparent that the metabolism of ribonucleosides by intact cells could not be explained adequately in terms of simple diffusion and phosphorylase action. Experimental observations have invited explanation in rather speculative terms such as biochemical compartmentation, surface-located reactions and ribosyl transferring enzymes. Accordingly, it was considered important to examine in some detail the metabolism of naturally-occuring purine ribonucleosides in intact cells.

A). Adenosine Metabolism

Adenosine may be metabolized in three directions, that is, by deamination to inosine, by cleavage to adenine, or by phosphorylation to AMP :



Adenosine deaminase is widely distributed in animal tissues (1,2,3,4) and has been demonstrated in tumor cells (5,6,7). The presence of this enzyme in Ehrlich ascites tumor cells is well established. Edmonds and LePage (8) found that Ehrlich ascites tumor cells, after incubation with adenosine, contained inosine. Straub et al. (9) reported that the adenosine deaminase activity of blood plasma of mice bearing the Ehrlich ascites tumor was 3 to 4 times higher than that of control animals, and also provided evidence that this was due to the release of this enzyme from the tumor cells. Paterson and Hori (10) reported that deamination of adenosine occured in cells of both the Ehrlich ascites tumor and its 6MP-resistant subline, and that the deamination activity was present only in the soluble fraction of these cells. They also reported that the inosine, so formed, was cleaved phosphorolytically to hypoxanthine and

ribose-1-phosphate (11). A purified cell fraction from the Ehrlich ascites tumor which catalyzed both the deamination of adenosine and the subsequent phosphorolysis of inosine has been described by Gotto et al. (12)

The direct cleavage of adenosine may take place in two ways, by hydrolysis or by phosphorolysis. Both have been described for bacterial and yeast cells (13,14). In lingcod muscle, adenosine may be hydrolyzed by a non-specific hydrolase, which cleaves several purine ribonucleosides and cytidine (15). The existence of adenosine phosphorylase has been demonstrated in beef liver (16) and in lingcod muscle (17). However, adenosine phosphorylase does not appear to occur in Ehrlich ascites tumor cells for these reasons: (a) adenine was not a ribosyl acceptor in a tumor cell system which synthesized inosine, thioinosine, and uridine when the appropriate substrates were present (base plus a "donor" ribonucleoside) (18), and (b) adenosine did not serve as a ribosyl "donor" in this system as will be seen in this report.

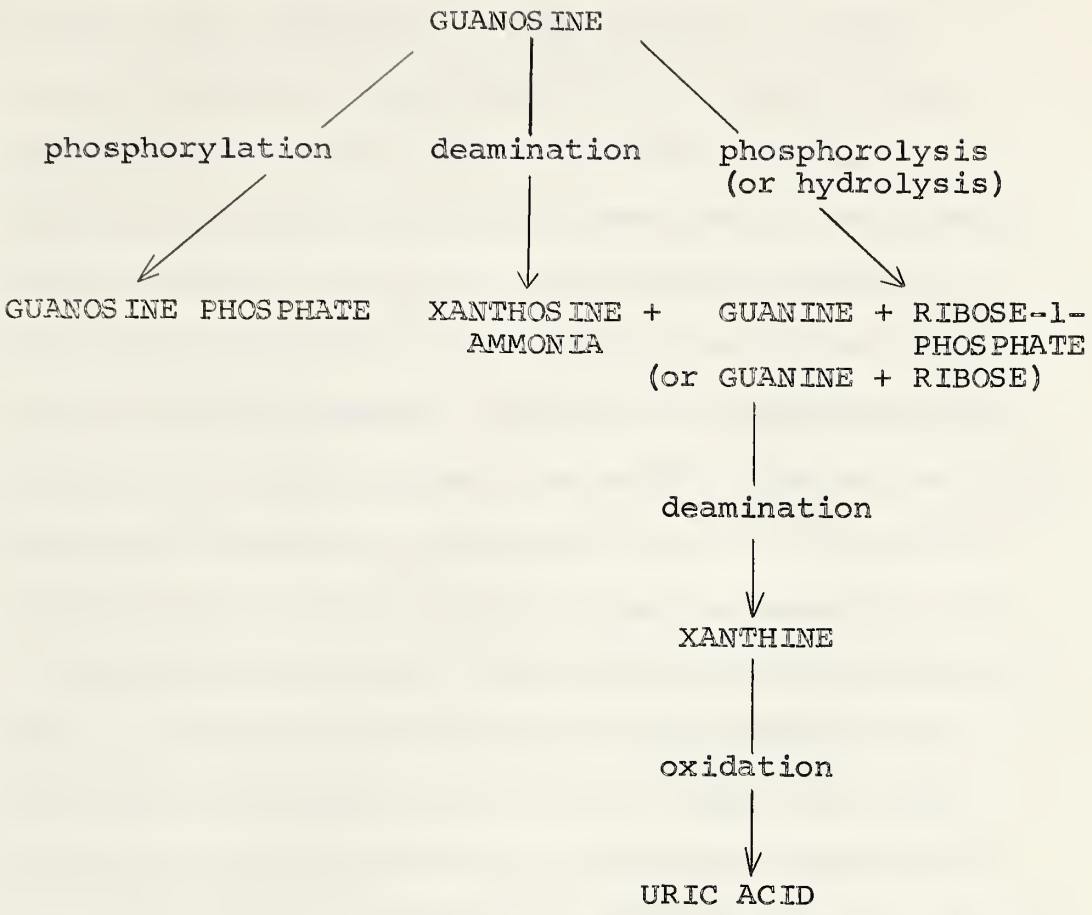
Adenosine kinase, which catalyzes the phosphorylation of adenosine, has been reported to be present in yeast (19,20), in the kidney and liver tissues of rats and rabbits (19), and in pig cardiac muscle (21). That adenosine kinase is also present in Ehrlich ascites

tumor cells may be seen in the following evidence. LePage and Edmonds (8) reported that inosine diluted the incorporation of glycine-2-^{14}C by nucleic acid adenine of Ehrlich ascites tumor cells, but did not so affect the incorporation of adenosine-8-^{14}C. This observation implied that adenosine was converted to AMP by direct phosphorylation rather than by a route involving hypoxanthine. Paterson and Hori (10) demonstrated that adenosine was more effective than inosine in diluting incorporation of radioactivity from hypoxanthine-8-^{14}C into the soluble adenine nucleotides of Ehrlich ascites tumor cells, a finding which can also be interpreted to mean that adenosine kinase is active in these cells. Recently it was found in this laboratory that 6-(methylmercapto)purine ribonucleoside is converted to the 5'-monophosphate derivative by these tumor cells, apparently by direct phosphorylation (22).

Thus, the metabolic fate of adenosine will vary with the tissue. In Ehrlich ascites tumor cells adenosine is deaminated to inosine and phosphorylated to AMP, but it is probably not cleaved to adenine.

B). Guanosine Metabolism

As with adenosine, there are three metabolic routes open to guanosine:



Deamination of guanosine has been demonstrated in animal tissues and in microorganisms (23,24,25,26,27). Most of these studies measured ammonia production. Guanosine deaminase, devoid of other enzymatic activities, has not yet been prepared. Deamination of guanosine by Ehrlich ascites tumor cells has not hitherto been reported.

As shown above, the cleavage of guanosine can be hydrolytic to yield free ribose, or phosphorolytic to yield ribose-1-phosphate. The former reaction has

been recorded for yeast (14), bacteria (13), and fish muscle (15). Guanosine phosphorolysis is known to occur in various animal cells (28). Purified preparations of purine ribonucleoside phosphorylase usually catalyze cleavage of both inosine and guanosine; however, it is not clear whether cleavage of these two compounds is catalyzed by a single enzyme or by separate enzymes. Yamada (29) has prepared a nucleoside phosphorylase from rabbit bone marrow which catalyzed cleavage of guanosine but not of inosine, suggesting that there may be separate guanosine and inosine phosphorylases. The cleavage of guanosine by Ehrlich ascites tumor cells has been reported by Paterson and Sutherland (11), who found that upon incubation with these cells, guanosine rapidly disappeared from the incubation medium and the free base appeared. Gotto et al. (12) prepared a purified cell fraction from Ehrlich ascites tumor cells which cleaved guanosine, inosine and xanthosine, the latter at a reduced rate. There is indirect evidence which indicates that cleavage of guanosine by intact Ehrlich cells is also phosphorolytic rather than hydrolytic. Paterson (18) showed that the synthesis of extracellular uridine and inosine in this system, which is apparently catalyzed by the coupled actions of ribonucleoside phosphorylases, is supported by guanosine but not by

ribose. Further, guanosine, but not ribose, is a good precursor of PRPP in these cells (30), indicating that the cleavage product is ribose-1-phosphate, which would be converted to PRPP by way of ribose-5-phosphate.

Guanine is deaminated by guanase to become xanthine. This enzyme is widely distributed in animal tissues (31,32). The presence of guanase activity in Ehrlich ascites tumor cells was recognized by Moore and LePage (33) who demonstrated that 6-thioguanine was converted to 6-thioxanthine and 6-thiouric acid in these cells. The latter product was formed at a very low rate.

Adenosine kinase has been extensively investigated, but few studies have been made of the enzymatic phosphorylation of guanosine. Guanosine phosphorylation has been demonstrated in fish milt extract (34); however, activities of other enzymes such as nucleoside phosphorylase and nucleotide pyrophosphorylase also appeared in the extract. Although guanosine kinase activity in Ehrlich ascites tumor cells has not yet been studied, evidence for the phosphorylation of deoxyguanosine by these cells may be found in the report by Overgaard-Hansen (35).

C). Metabolism of Ribose-1-Phosphate Derived from Adenosine and Guanosine.

It has been stated above that adenosine is not phosphorolytically cleaved by Ehrlich ascites tumor cells, but the deamination product is converted to hypoxanthine

and ribose-1-phosphate. In contrast, guanosine is directly phosphorolyzed by these cells. The cleavage product, ribose-1-phosphate may be further metabolized. Paterson and Sutherland (11) have shown that ribose moieties of ribonucleosides are converted to lactate. Ribose-1-phosphate participates in ribonucleoside synthesis by reversal of the phosphorolytic reaction (18) and in nucleotide synthesis after conversion to PRPP (30,36) in these tumor cells.

II. MATERIALS AND METHODS

A). Tumors

The Ehrlich ascites carcinoma used in these experiments is a hypotetraploid line (73-74 chromosomes[1]) which has been maintained since 1956 by weekly transplantation in Swiss mice (37). The growth of this tumor line is severely inhibited by treatment with 6MP or Me-6MPR. A 6MP-resistant subline of this tumor was developed by the serial transplantation of 6MP-treated cells (38). This line, now in its 404th transplant generation is cross-resistant to Me-6MPR, differs from the parent line in chromosome number (68-69[1]) and in two aspects of nucleotide metabolism (10,39). However, the two lines do not appear to differ in ability to carry out nucleoside cleavage, synthesis and exchange reactions nor in the sensitivity of these reactions to inhibition by Me-6MPR[2].

B). Preparation of Cells.

The cells were collected 6-8 days after transplantation. Ascitic fluids were drained through abdominal incisions into tared 40 ml conical centrifuge tubes and were mixed with 3-4 volumes of cold physiological saline. Red blood cells were removed from the suspension by centrifuging

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1. H. Kirk and S.B. Hrushovetz, University of Saskatchewan.
2. A.R.P. Paterson and A.I. Simpson, unpublished results.

at 600 x g for 40 seconds; the supernatant, which contained most of the red blood cells, was discarded. The volume of the suspension was made up to 40 ml with saline and the low speed centrifugation repeated to remove more erythrocytes. Usually 4 or 5 centrifugation steps were employed. Finally, the cells were packed at 2,400 x g for 10 minutes, and after the tube was wiped dry, the wet weight of the cell pack was measured. The cells were suspended in a volume of cold Krebs Ringer phosphate medium (40)[3] equal to 4 times the weight of cells. Sonified cells were prepared by treating the cell suspension with a 20 kc probe type sonic oscillator. By successive short periods of sonic treatment and by employing phase contrast microscopy to examine the cell suspension after each treatment, cell breakage was accomplished with a minimum exposure to sonic oscillation. A total of 50 seconds sonic treatment was usually adequate.

C). Reaction Conditions.

Incubation mixtures were prepared in duplicate in Krebs Ringer phosphate medium and, except where noted, contained 0.1 g cells (wet weight) per ml reaction mixture. Unless otherwise specified, incubation mixtures were 1.0 ml in volume and were contained in 10 ml beakers.

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3. The concentration of calcium was reduced to one third.

Incubations were conducted at 37°C in a shaking water bath (100 strokes per minute) with the air as the gas phase; unless otherwise specified the incubation period was 15 minutes.

D). Termination of Reactions and Preparation of Samples

1. Adenosine metabolism.

In these experiments, reaction mixtures were chilled in ice water after incubation, and incubation mixtures were either treated directly with perchloric acid or cells and medium were treated with perchloric acid separately after separation by centrifugation at 2,300 x g for 2 minutes at 4°C. The perchloric acid extracts were clarified by centrifugation.

2. Guanosine Metabolism.

When medium fractions were to be analyzed, for guanosine and its metabolites, reaction mixtures were not chilled to terminate the incubation, but were centrifuged at room temperature in order to avoid precipitation of guanine which has a very low solubility. Medium samples thus obtained, were then heated in boiling water for 2 minutes and clarified by centrifugation at room temperature. In other experiments in which whole reaction mixtures were analyzed, the reactions were terminated and samples for analysis were prepared as in the adenosine metabolism experiments.

E). Perchloric Acid Extraction of Cells, Medium, and Complete Reaction Mixtures.

Cold 22% perchloric acid was added to chilled incubation mixtures or to the medium samples obtained therefrom to make the final concentrations 2%. To extract cells, 0.4 ml portions of 2% cold perchloric acid were added to the packed cells obtained from 1.0 ml samples of reaction mixtures. The perchloric acid mixtures were allowed to stand for at least 15 minutes in cold before the denatured protein was removed by centrifugation.

F). Determination of Extracellular Water, Intracellular Water, and Total Water of Packed Cells.

In the analysis of perchloric acid extracts of tumor cells in one experiment (Table III), it was necessary to account for substances contributed by medium trapped in the cell pellet. This was done by determining in model experiments the extracellular water content of cell pellets prepared under the same experimental conditions. With this information, analysis of the medium permitted corrections to be made for medium components present in the cell extracts.

Extracellular water was measured by determining the ^{14}C content of cell pellets recovered from medium which contained ^{14}C-sucrose. Sucrose does not readily enter

animal cells and has been widely used to determine extracellular space (for example, see refs. 41 and 42). The relationship between the volume of extracellular water and the total pellet water was determined on cells which had been incubated under experimental conditions duplicating those used in experiments on adenosine metabolism.

The following experiment was repeated 4 times and the results are summarized in Figure 1. A large volume (40 ml) of the usual incubation mixture containing adenosine (2m$\underline{M}$) was incubated in 250 ml Erlenmeyer flasks under usual conditions for 10 minutes and then chilled in ice water. Measured samples with different volumes (1-8 ml) were placed in the usual 12 ml conical centrifuge tubes. The rest of the incubation mixture was poured into a 150 ml Erlenmeyer flask which contained ^{14}C-sucrose (5×10^5 c.p.m., approximately 0.05 mg). After mixing, measured volumes of the mixture were placed in centrifuge tubes as above. All tubes were centrifuged at 2,300 x g at 4°C for 2 minutes. Tubes which did not contain radioactive sucrose were drained and their surfaces above the cell pellets were wiped dry. These tubes were then weighed and their contents dried at 105°C to a constant weight. The change in weight upon drying was taken as the total water content of the cell pellet (W_t).

The supernatant fluids from tubes which contained ^{14}C-sucrose were reserved for analysis as follows: measured samples were made 2% in perchloric acid as described previously and, after removal of precipitated protein, were assayed for radioactivity (R_m). Cells from these tubes were suspended in a measured volume of 2% perchloric acid (V_a). After standing for 15 minutes in the cold, the extract was clarified by centrifugation and samples were taken for assay of radioactivity (R_c).

Calculation:

$$\text{Volume of extracellular water in cell pack } (W_{ex}) = \frac{[\text{Total water } (W_t) + \text{Vol. of PCA added } (V_a)] \times \text{c.p.m. per ml} (R_c)}{1.1 \times \text{c.p.m. per ml PCA-treated medium } (R_m)}$$

$$\text{Volume of intracellular water in cell pack } (W_{in}) = \text{Total water } (W_t) - \text{Volume of extracellular water } (W_{ex}).$$

Thus, cell pellets of different sizes were analyzed for extracellular water and total water; a linear relationship was found between these parameters as seen in Figure 1. Since data from four replicate experiments fitted this straight line quite well, it was considered valid to apply this relationship to other experiments. From these experiments it was also found that 1.00 gram of packed cells (wet weight) contained 0.73 ml of total water, of which 0.33 ml was intracellular water and 0.40 ml was extracellular water.

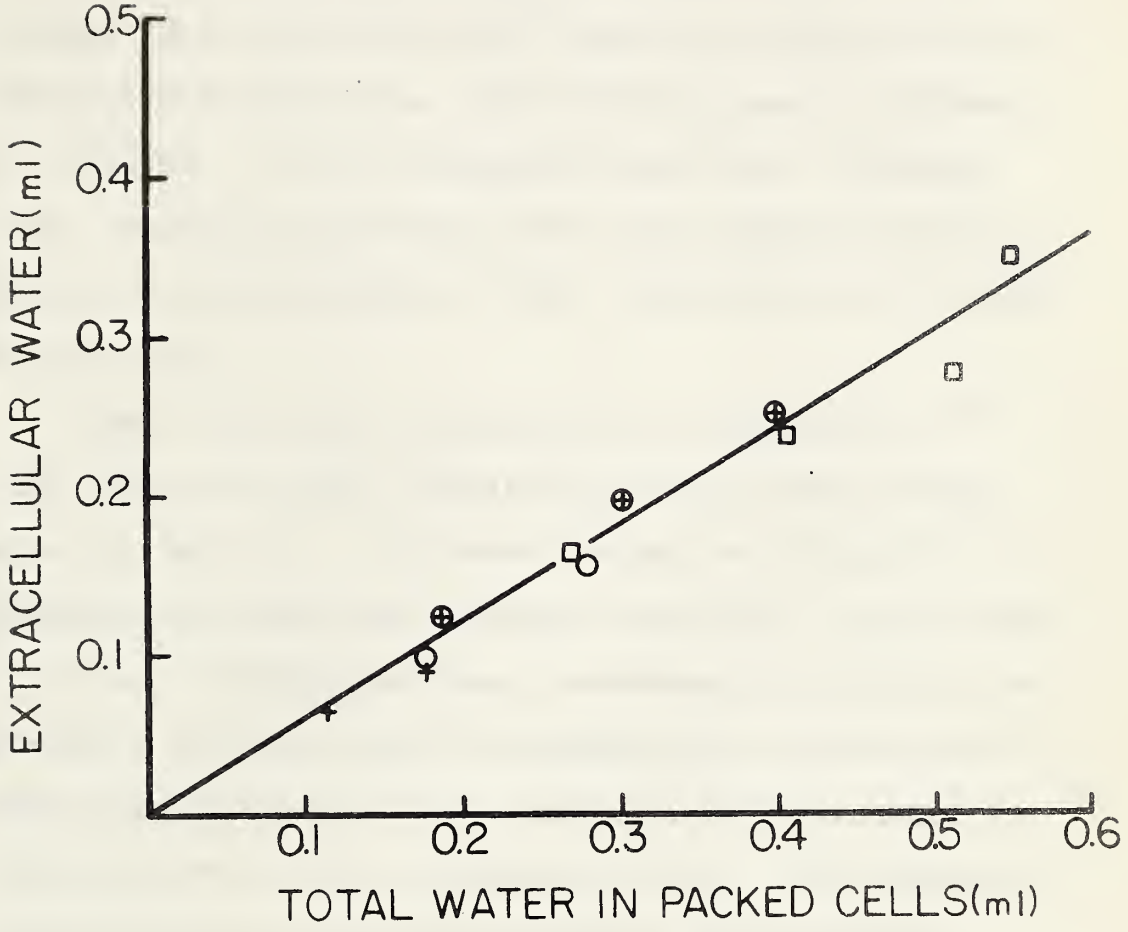

FIG. 1. The relationship between total water and extracellular water in packed Ehrlich ascites tumor cells collected by centrifugation at 2,300 x g for 2 minutes.

O Experiment 1, ⊕ Experiment 2, + Experiment 3,

□ Experiment 4.

G). Determination of Intracellular and Extracellular Inosine Concentrations.

In order to determine the distribution of inosine between tumor cells and their supporting medium during metabolism of adenosine, the following special procedure was devised. In this procedure cells were collected under conditions identical with those employed above in determining extracellular water, making the use of this factor valid.

Tumor cells were incubated with adenosine-8-^{14}C (2mM) under the usual conditions for the time periods shown in Table II. Duplicate incubation mixtures were prepared for each time point and each had a final volume of 6.0 ml. Incubations were terminated by chilling the flasks in ice water and two samples (2.5 ml) from each flask were placed in 12 ml conical centrifuge tubes and spun at 2,300 x g for 2 minutes at 4°C. The supernatant fluids from each flask were pooled and reserved for analysis (see below). The tubes with their pellets of packed tumor cells were drained and wiped dry. One was used for the measurement of the total cell water (see preceding section) and the other was extracted with 2% perchloric acid.

A portion of each supernatant fluid was made 2% in perchloric acid as described previously; after clarification, measured portions of these extracts and of the

cell extract were analyzed for their inosine content by paper chromatography.

Calculations:

Let the total water content (ml) of the cell pellet be W_t; let the inosine concentration (m$\underline{M}$) in the perchloric acid extract of the cell pellet be $[HR]_c$ and that of extracellular medium be $[HR]_m$; let the extracellular and intracellular water content (ml) be W_{ex} and W_{in} respectively; then,

the intracellular inosine concentration will be equal to

$$\left[\frac{(1.0 + W_t) \times [HR]_c - W_{ex} \times [HR]_m \times 1.1}{W_{in}}\right] m\underline{M}$$

H). Assay of Reaction Products.

The rates of inosine or guanosine utilization were measured by the disappearance of orcinol-positive material (43) from the incubation medium. In other experiments, a previously described method (18), which combines the use of isotopic substrates and chromatographic (or electrophoretic) isolation of products, was employed to assay metabolic transformations amongst the ribonucleosides. To illustrate this procedure, the assay of inosine synthesis is described: after tumor cells were incubated with hypoxanthine-8-^{14}C and a non-radioactive ribonucleoside, medium samples (usually 0.010 ml) were applied to paper chromatograms

along with "carriers" (non-isotopic inosine and hypoxanthine). The chromatograms were developed in solvent E (see p. 20) and carrier spots were cut out for ^{14}C assay by the liquid scintillation technique. The sum of the radioactivities accompanying the carrier spots was equal to the activity of an identical sample applied to a portion of the chromatogram that was left untouched by the developing solvent. The concentrations of products and the remaining substrate were calculated from the volume of sample chromatographed, the observed radioactivities of the carrier spots, and the initial specific activity of the radioactive substrate. Similar procedures were employed to follow the metabolism of adenosine, guanosine, and uridine. In electrophoretic analyses, non-isotopic carriers were also employed to locate products. In each instance, the products and substrate remaining accounted for essentially all of the radioactivity applied to the chromatogram or electrophoretogram.

In studying the metabolism of guanosine-8-^{14}C, chromatograms and electrophoretograms were cut into lanes which extended from the origin to the front and the lanes were then cut into 1 cm transverse segments which were assayed individually for radioactivity.

Paper chromatogram segments or areas of carriers on paper were assayed directly for the radioactivity by

the liquid scintillation counting procedure of Wang and Jones (44). Paper samples were inserted into counting vials which contained counting fluid (PPO-POPOP-toluene (45)) and were counted in a Nuclear-Chicago Model 725 liquid scintillation system.

I). Electrophoresis.

In separating the metabolic products of guanosine, electrophoretograms were run for 6 hours in 1.0 M sodium borate buffer, pH 7.4, at a potential of 22 volts per cm, using Whatman 3 MM paper. The following compounds are arranged in order of increasing mobility towards the anode in this system: guanine, xanthine, guanosine, xanthosine, GMP, GDP, GTP. The first four compounds were separated well from each other, but the last four compounds overlapped.

J). Paper Chromatography

The following solvent systems were used in various parts of these studies.

Solvent A: isoamyl alcohol and aqueous 5% disodium phosphate, mutually saturated and used in equal volumes (46).

Solvent B: aqueous ammonium bicarbonate, 85% saturated (47).

Solvent C: water adjusted to pH 10 with NH_4OH (48).

Solvent D: isobutyric acid-concentrated ammonium hydroxide - water (66 : 1: 33, v/v) (49)

Solvent E: n-butanol - glacial acetic acid - water (5: 3 : 2, v/v) (18).

Solvent F: isobutyric acid - water - concentrated ammonium hydroxide - 0.1 M versene (100: 55. 8 : 4.2 : 1.6, v/v) (50).

Solvent G: 5 M ammonium acetate (pH 9.0) - saturated sodium tetraborate - ethanol - 0.5 M versene (20 : 80 : 180 : 0.5, v/v) (51,52).

K). Compounds

1. Adenosine-8-^{14}C

This compound was either a commercial product or was prepared from adenine-8-^{14}C by the following procedures:

Adenine-8-^{14}C was converted to AMP-8-^{14}C enzymatically, using extracts of ascites cells as a source of adenylate pyrophosphorylase. The adenylate so formed was dephosphorylated with prostatic phosphomonoesterase. Packed cells (4 ml) were suspended in 24 ml 0.2 M tris buffer, pH 8.0. The cell suspension was sonified, centrifuged at 100,000 x g for 60 minutes at 4°C, and diluted with 6 volumes of the tris buffer. A reaction mixture (10 ml)

containing the following components was prepared:

PRPP, 30 μmoles; adenine-8-^{14}C (specific radioactivity, 6.8 x 10^6 c.p.m. per μmole), 20 μmoles; Mg^{++}, 60 μmoles; tumor cell extract, 2 ml. This reaction mixture was incubated at 37°C for 40 minutes with shaking. The reaction was terminated by heating in boiling water for 2 minutes, and then centrifuged to remove denatured protein. Paper chromatography in solvent E showed that 45% of the original adenine was converted to nucleotide. The AMP product was isolated by ion exchange chromatography using a 1 x 15 cm column of Dowex-1X8 (200-400 mesh, formate form) which was eluted with a linear gradient system that contained in identical mixing and reservoir vessels, 425 ml water and 406 ml 0.31 M formic acid, respectively. The eluate was collected in 5 ml fractions and adenine was found in fractions 9 to 20 inclusive, and AMP was in fractions 54 to 76. The AMP fractions were pooled, lyophilized, and dissolved in 10 ml of water. Paper chromatography in solvent A showed that 30% of the original adenine was recovered as AMP.

In the next step AMP was dephosphorylated by prostatic phosphatase. To the above AMP solution was added 5 ml of 0.2M acetate buffer, pH 5.4, then incubated at 37°C for 3 hours with 5 ml prostatic phosphatase[4]. After

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4. Prepared by Dr. A. R. P. Paterson by the procedure of Kerr and Chernigoy (53).

incubation, perchloric acid was added to a final concentration of 2%. The mixture was clarified by centrifugation and neutralized with 22% potassium hydroxide. After standing at 4°C overnight, the precipitate was removed by centrifugation. Paper chromatography by solvent A showed that 60% of the AMP was hydrolyzed to adenosine.

The charcoal adsorption method of Tsuboi(54) was employed to separate adenosine and AMP from the above mixture and yielded these compounds in a salt-free aqueous solution which was then put onto a 1 x 6 cm column of Dowex-1-formate. Adenosine was eluted with 75 ml of water, leaving AMP on the column. Adenosine-containing eluate fractions were pooled, lyophilized and then redissolved in a small volume of water. The adenosine product was shown to be more than 99% pure by paper chromatography in two solvent systems, A and B. Approximately 20% of original adenine-8-^{14}C was recovered as adenosine-8-^{14}C.

2. Other compounds.

Other ^{14}C-labelled compounds and naturally occuring ribonucleosides were commercial products. Me-6MPR was obtained from the Cancer Chemotherapy National Service Center, Bethesda, Maryland.

III RESULTS

A). Adenosine Metabolism

1. Appearance of inosine in the extracellular medium during in vitro adenosine metabolism.

When intact Ehrlich ascites tumor cells were incubated with adenosine-8-^{14}C, it was found that inosine and hypoxanthine appeared in the incubation medium and that no significant amount of adenine was formed. This result, which has been confirmed in a number of experiments, is illustrated in Figure 2. Shown is the distribution of radioactivity on a paper chromatogram which resolved adenosine-8-^{14}C and its metabolites. This particular solvent system (D) does not completely separate inosine and AMP; however, when paper chromatograms of this sample were run again in the second dimension with solvent E, it was found that no significant amount of radioactivity accompanied the AMP carrier. Paper chromatography in solvent B and C also demonstrated the appearance of inosine in the medium. Similar results were also obtained with cells of the 6MP-resistant Ehrlich subline.

In the experiment of Table I, adenosine-8-^{14}C was incubated with tumor cells and at several time intervals the incubation was analyzed. It is apparent that as adenosine disappeared there was a progressive accumulation of inosine and hypoxanthine in the medium.

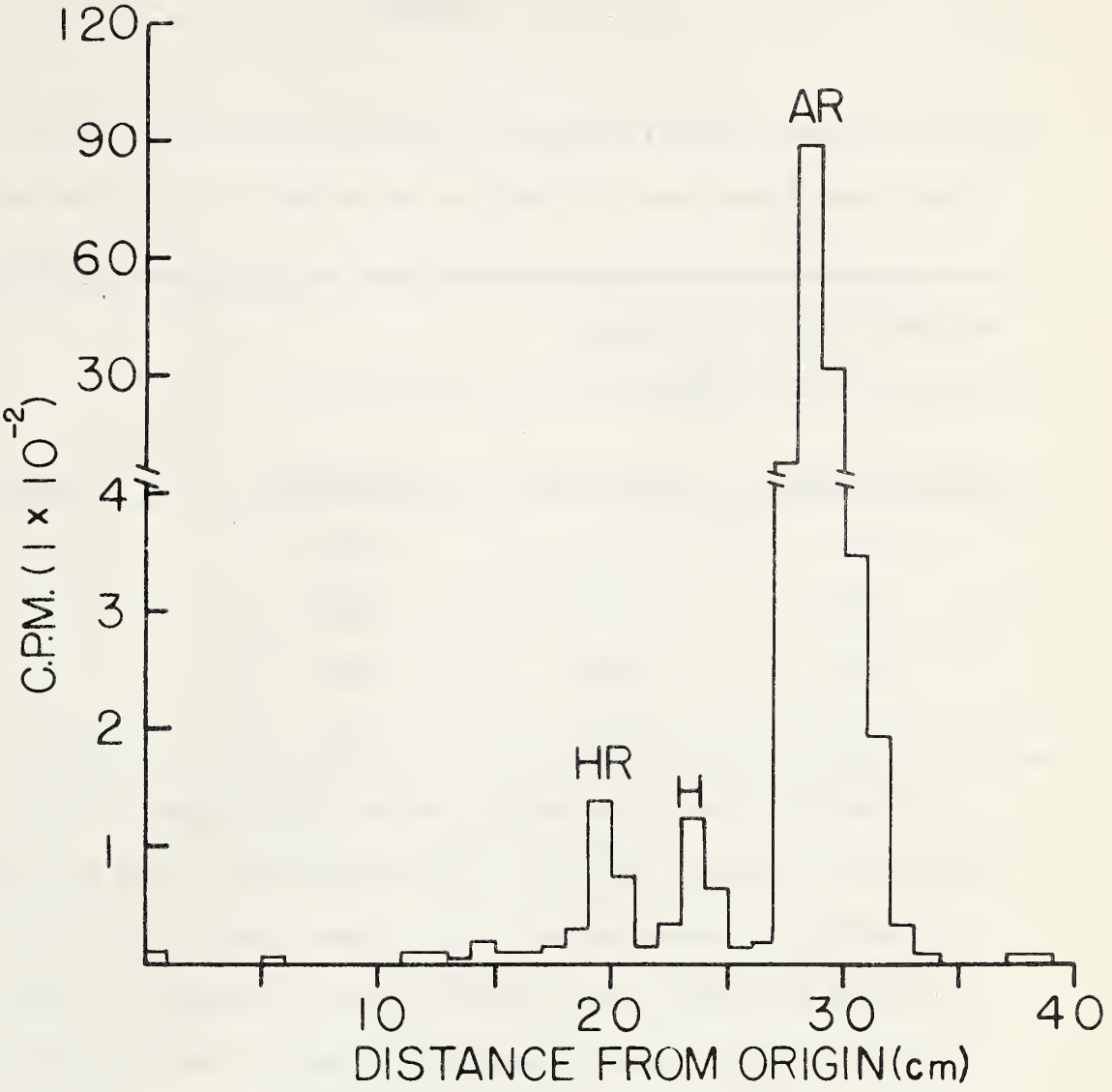

FIG. 2. The formation of extracellular inosine during the incubation of ascites cells with adenosine.

Tumor cells were incubated under the usual conditions as described in "Materials and Methods" with adenosine-8-^{14}C (2 m$\underline{M}$; specific activity, 2.94 x 10^5 c.p.m. per μmole). After incubation, the medium fraction was treated with perchloric acid and a 0.025 ml sample was chromatographed in solvent D with carriers. Transverse segments(1 cm) of the chromatogram were assayed for radioactivity. Peaks of radioactivity were identified by their coincidence with carriers.

TABLE I

Time course of the formation of extracellular inosine during the metabolism of adenosine by Ehrlich ascites tumor cells in vitro

Time (Minutes)	Concentration in medium (mM, x 10^3)		
	Adenosine	Inosine	Hypoxanthine
0	530	6.4	3.4
20	328	15.7	34.8
40	185	24.4	67.2
60	81	34.2	111.1

NOTE: Tumor cells were incubated under the usual conditions with adenosine-8-^{14}C (0.5 mM; specific activity, 8.25 x 10^5 c.p.m. per μmole) for the periods indicated in the table. Samples of medium from each reaction mixture were prepared and chromatographed as described in Figure 2. Concentrations of adenosine and its metabolites were calculated from the radioactivity accompanying the carriers and the initial specific activity of adenosine-8-^{14}C.

Since inosine is known to be readily catabolized by these cells (11,12), the appearance of inosine in the medium seemed to be unusual and to warrant further study.

2. Rates of inosine phosphorolysis and adenosine deamination.

It was considered that the relative rates of inosine cleavage and adenosine deamination might afford an explanation for the appearance of extracellular inosine. If inosine was produced faster than it could be catabolized, it would accumulate inside the tumor cells and subsequently diffuse outwards into the medium. To test this possibility, the rates of inosine phosphorolysis and adenosine deamination were compared. The experiment of Figure 3 shows the effect of concentration on the rate of disappearance of inosine from the medium. As can be seen, this rate was maximal at about 22 μmoles per gram of cells per hour, when the initial inosine concentration was 4 mM. At the initial concentration of 2mM, the rate of disappearance of inosine was 19 μmoles per gram of cells per hour. At the same concentration, the rate of adenosine disappearance from the reaction mixture was 6.5 ± 0.7[5] μmoles per gram of cells per hour. This is an averaged value obtained from Tables VI and VIII. It should be noted that

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5. Average deviation from the mean.

the rate of disappearance of inosine from the medium might not be the same as the rate of inosine phosphorolysis, because the former value would include intracellular inosine. However, since the volume of intracellular water was only about 3% of that of the entire reaction mixture (see "Materials and Methods"), the difference between these two values would probably be small[6], presuming that there is no large concentrative uptake of inosine. It also should be noted that the rate of adenosine disappearance from whole reaction mixture might be higher than the rate of deamination since the direct phosphorylation of adenosine takes place in these cells (see "Introduction") and would contribute to the rate of disappearance of adenosine. From these data, it is apparent that the tumor cells are able to phosphorolyze inosine at a rate at least three times higher than that at which they can deaminate adenosine, and yet, despite this fact, inosine appears in the incubation medium.

3. The extracellular deamination of adenosine

Since Ehrlich ascites tumor cells *in vitro* release enzymes into their incubation medium (18,55,56), it was considered possible that the appearance of extracellular

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6. In a similar experiment, Paterson and Sutherland (11) found that Ehrlich ascites tumor cells cleaved inosine (initial concentration 2mM) at the rate of 19.2 μmoles per gram of cells per hour by measuring hypoxanthine appearing in the medium using the xanthine oxidase procedure.

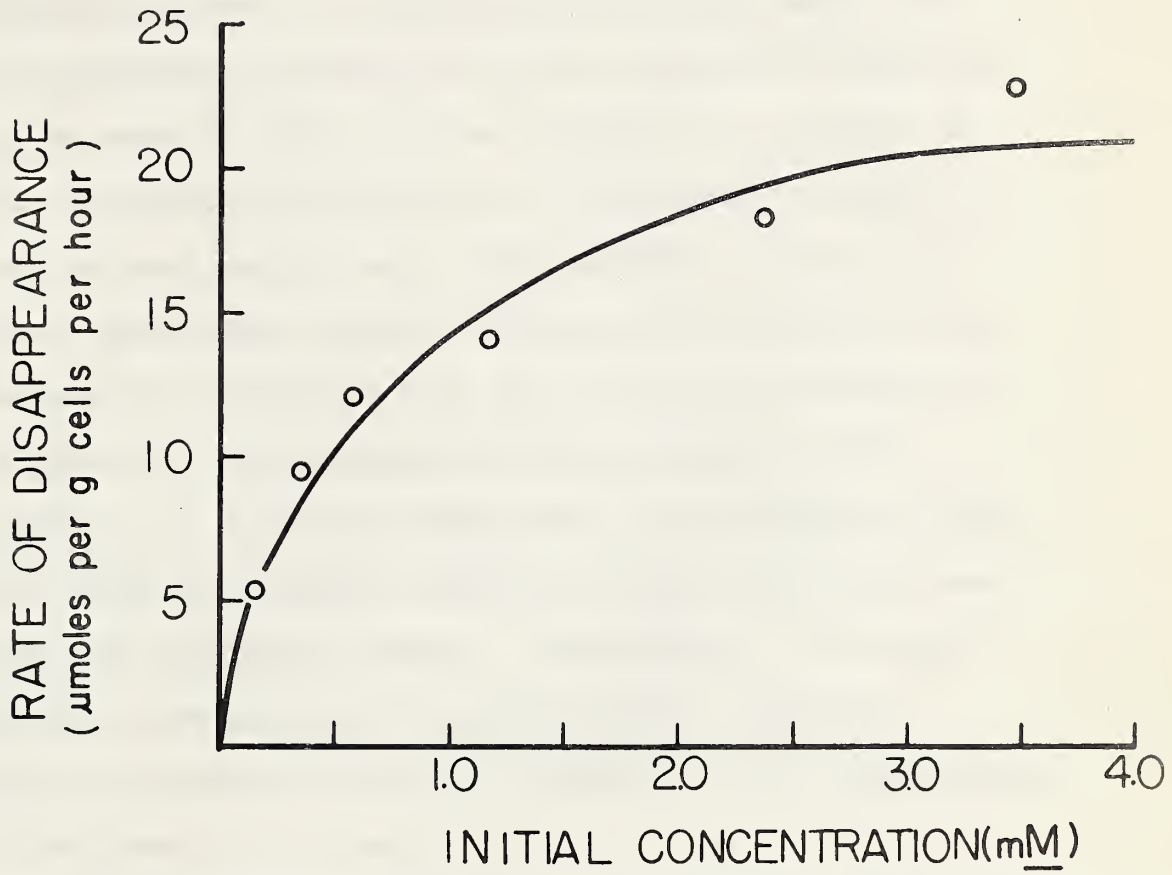

FIG. 3. Effect of concentration on the rate of inosine utilization by Ehrlich ascites tumor cells.

Tumor cells were incubated under the usual conditions with inosine at the concentrations indicated. Samples of the incubation mediums were analyzed for orcinol-positive material using AMP as a standard; the disappearance of orcinol-positive material was regarded as inosine utilization.

inosine was due to the presence of adenosine deaminase in the medium. To test this possibility, adenosine was incubated with intact cells and "postincubation medium", which was prepared by incubating tumor cells under the usual experimental conditions in the absence of substrate. As can be seen in Table II, the formation of inosine in the postincubation medium was only 39% of that found in the medium when intact cells were present. Since the adenosine deaminase content of the medium would probably increase with incubation time (as did that of enzymes of the glycolytic and pentose phosphate pathways (55)), the activity of adenosine deaminase in the medium of the usual incubation mixtures should be lower than that found for the postincubation medium. Accordingly, it is concluded that the activity of extracellular adenosine deaminase accounted for only a fraction of the accumulation of the extracellular inosine.

4. Time course of inosine formation during the metabolism of adenosine.

It was felt that a time study of intracellular and extracellular inosine concentrations during the metabolism of adenosine by the tumor cells might help to explain the anomalous appearance of extracellular inosine.

In the experiment of Table III, adenosine-8-^{14}C was incubated with tumor cells and at various time intervals, the concentrations of inosine inside and outside the cell

TABLE II

Is adenosine deaminase released into the incubation medium?

Test system	Inosine concentration in medium (mM, x 10^2)
Complete incubation mixture	3.48
"Postincubation medium" only	1.36

NOTE: Tumor cells were incubated under the usual conditions with and without adenosine-8-^{14}C (2mM; specific activity, 2.94 x 10^5 c.p.m. per μmole). After incubation, cells were removed from both types of mixture. Medium samples from those without substrate ("postincubation medium") were reincubated for the same period with adenosine-8-^{14}C. Measured samples of both types of medium were analyzed as described in Table I.

were measured. Table III shows that intracellular inosine concentrations were several times higher than extracellular concentrations during the entire course of the incubation. This type of distribution would be expected if the deamination of adenosine took place inside the cell and the product diffused outward.

5. Effect of Me-6MPR on adenosine metabolism

It has been found in this laboratory that Me-6MPR inhibits the metabolism of several ribonucleosides in Ehrlich ascites tumor cells, and that this inhibitory action is manifested only towards intact cells (57). It is our working hypothesis that Me-6MPR inhibits ribonucleoside metabolism by blocking the entrance of these compounds into the cell, which, by implication, appears to be enzymatic process. If this mechanism of inhibition is valid, then it is expected that Me-6MPR could inhibit the metabolism of a variety of nucleosides. To test this possibility, the effects of this compound on adenosine metabolism were studied.

In the experiment of Table IV, tumor cells, rather than the medium, were analyzed for metabolites of adenosine. Cells were incubated with adenosine as usual, washed twice with cold medium and were then extracted with cold perchloric acid. Two paper chromatographic systems were used to analyze the perchloric acid extracts. By chromago graphy in solvent D, adenosine, hypoxanthine, inosine, and

TABLE III

A time study of inosine formation during the metabolism of adenosine by Ehrlich ascites tumor cells in vitro.

Incubation period (minutes)	Inosine concentration (mM, x 10^3)	
	Intracellular	Extracellular
0	0.0	1.5
5	15.9	4.8
15	28.6	10.8
30	57.5	16.4
60	123.0	35.5

NOTE: Tumor cells were incubated under the usual conditions with adenosine-8-^{14}C (1mM; specific activity, 2.96 x 10^5 c.p.m. per μmole). Duplicate incubation mixtures (6ml) were prepared for each incubation period and were processed under carefully defined conditions (see "Materials and Methods") to obtain cell and medium fractions. These were treated with particular volumes of perchloric acid and the resulting extracts were analyzed by paper chromatography for their inosine content. Since the relationship between extracellular space and total water content in cell pellets obtained under these conditions had been determined prior to these experiments (see Figure I), a correction was applied to the analytical values obtained for cells in order to compensate for inosine introduced into the cell pellet by trapped medium. Inosine was separated from other compounds by two dimensional paper chromatography (first dimension, solvent D; second dimension, solvent E).

nucleotides other than AMP were separated well from each other. However, AMP accompanied inosine in this system. Further, paper chromatography in solvent E separated adenosine and hypoxanthine from inosine and the nucleotides. Thus, by combining data obtained with these two paper chromatographic systems adenosine, inosine, hypoxanthine, and the combined nucleotides were determined. As may be seen in Table IV, Me-6MPR had only a small effect on the intracellular concentrations of adenosine and its metabolites, hypoxanthine and nucleotides; however, conversion to inosine was reduced by 50%. The effect of Me-6MPR on the appearance of extracellular inosine was also studied. Table V shows that Me-6MPR depressed the formation of extracellular inosine by about 30%.

Table VI shows the analogue ribonucleoside decreased the rate at which adenosine was metabolized by intact cells, but had no effect on that by sonified cells or by the postincubation medium. Thus, it is apparent that the inhibitory action of Me-6MPR on adenosine metabolism required the integrity of cells.

6. Adenosine and the synthesis of ribonucleosides.

Ribosyl transfer reactions between ribonucleosides and bases have been demonstrated for a number of cell types (18,58,59,60). In the experiments of Table VII the ability of adenosine to participate in such reactions was studied with intact tumor cells.

TABLE IV

Effect of Me-6MPR on adenosine metabolism in Ehrlich ascites tumor cells in vitro

Inhibitor	Concentration in PCA extract of cells (mM, x 10)			
	Adenosine	Inosine	Hypoxanthine	Nucleotides
None	1.36	0.109	0.111	2.64
Me-6MPR(2mM)	1.21	0.054	0.105	2.41

NOTE: Incubation mixtures (3 ml) contained 2 mM adenosine-8-^{14}C (specific activity, 2.94 x 10^5 c.p.m. per μmole). After incubation under the usual conditions, 2.5 ml samples were centrifuged and the supernatant was removed. The packed cells were resuspended in 10 ml of cold medium and were centrifuged again. This washing process was repeated again, then the tubes were drained and wiped to dryness. Perchloric acid extracts of the packed cells were analyzed for adenosine and its metabolites as described in the text.

TABLE V

Effect of Me-6MPR on the formation of extracellular inosine during adenosine metabolism by Ehrlich ascites tumor cells in vitro.

Inhibitor	Inosine concentration (mM, x 10^2)
None	3.48
Me-6MPR	2.37

NOTE: Substrate and inhibitor concentrations were same as described in Table IV. This experiment was performed at the same time as the experiment of Table II.

TABLE VI

Does Me-6MPR affect adenosine metabolism by broken tumor cells and by extracellular enzymes?

	Total mM of products formed (x 10)			
	Expt. A		Expt. B	
	Me-6MPR absent	Me-6MPR present	Me-6MPR absent	Me-6MPR present
Intact cells	1.46	1.23	1.99	1.28
Broken cells	2.19	2.16		
Postincubation medium	0.29	0.28		

NOTE: Tumor cells were incubated with adenosine-8-^{14}C in the presence or absence of Me-6MPR under the conditions as described in Table IV. Incubation mixtures (complete)were extracted with perchloric acid, and analyzed as described in Table I.

Uracil-2-^{14}C or hypoxanthine-8-^{14}C was incubated with tumor cells in the presence of adenosine or other ribonucleosides, and the medium fractions of the incubation mixtures were analyzed by paper chromatography. Table VII shows the results of these experiments. Guanosine and inosine supported the synthesis of radioactive uridine, but adenosine did not participate in this reaction. It is seen that an exchange took place between uracil-2-^{14}C and uridine to yield uridine-^{14}C. A similar exchange of isotope occured between hypoxanthine-8-^{14}C and inosine. Guanosine and uridine provided ribose for inosine synthesis, but again adenosine did not participate in the reactions.

7. Adenosine metabolism in 6MP-resistant tumor cells.

In Table VIII comparisons are made of the rate of adenosine metabolism by the Ehrlich ascites tumor and its 6MP-resistant subline. The resistant cells were found to have a significantly higher rate of adenosine metabolism. The meaning of this difference is not apparent.

8. Rate of adenosine metabolism by mouse erythrocytes.

Since Ehrlich ascites tumor cells are usually accompanied by red blood cells, it was, of course, desirable to evaluate the capacity of mouse erythrocytes to metabolize adenosine. Although all tumor cell preparations were carefully washed by the low speed centrifugation procedure

TABLE VII

Inability of adenosine to support the synthesis of extracellular inosine and uridine by Ehrlich ascites tumor cells.

Ribosyl donors	Concentration of nucleoside product in the medium (mM x 10)		
	Inosine		Uridine
	Expt. A	Expt. B	Expt. C
None	0.05	0.26	0.09
Adenosine	0.14	0.36	0.08
Inosine	0.85*	1.18*	0.38
Guanosine		1.10	0.19
Uridine		2.88	2.74*

NOTE: The ability of various nucleosides to support inosine or uridine synthesis was compared. In Expts. A and B, tumor cells were incubated with hypoxanthine-8-^{14}C (1 mM; specific activity, 2.3 x 10^6 and 1.82 x 10^5 c.p.m. per µmole in Expts. A and B, respectively) and a ribonucleoside substrate (2mM). As uridine synthesis was measured in Expt. C, the labelled base was uracil-2-^{14}C (2mM ; specific activity, 1.2 x 10^6 c.p.m. per µmole). The usual reaction conditions were employed except in Expt. B, where a bicarbonate-buffered medium (61) without glucose and a gas phase of oxygen-carbondioxide (5%) were used. Product concentrations in the medium were determined by the usual chromatographic procedures using solvent E for inosine, and solvent G for uridine.

*In these instances, the incorporation of isotope by the nucleoside is the result of an exchange reaction between the nucleoside and the ^{14}C-labelled base. The values noted above represent the amount of radioactive bases appearing in the form of nucleoside, but do not represent net synthesis.

TABLE VIII

Rate of adenosine metabolism by cells of the Ehrlich ascites tumor and its 6MP-resistant subline.

Cells	Total metabolites formed in whole incubation mixture (m$\underline{M}$, x 10)	
	Expt. A	Expt. B
Ehrlich ascites carcinoma	1.41	1.76
6MP-resistant subline	2.71	2.20

NOTE: Experimental conditions were the same as described in Figure 2. Incubation mixtures (complete) were extracted with perchloric acid, and analyzed as described in Table I.

to minimize contamination by erythrocytes, small numbers were undoubtedly present in all tumor preparations.

Adenosine-8-^{14}C was incubated with the red cells and, at various time intervals the complete incubation mixtures (cellsplus medium) were analyzed. Since it had been demonstrated with paper chromatography in solvent E that nucleotides were minor reaction products, paper chromatography in solvent D was judged to be adequate for analysis of reaction products although this solvent did not resolve AMP and inosine. Table IX shows that deamination of adenosine by these red cells was a very rapid process and that formation of inosine far exceeded hypoxanthine production. The rate of adenosine deamination (expressed as the sum of inosine and hypoxanthine formed) during the first 15 minutes of incubation was 47 μmoles per ml of packed cells per hour. These data demonstrate that contamination by erythrocytes could greatly distort assessments of adenosine metabolism by tumor cells.

B). Guanosine Metabolism

1. Metabolic fate of guanosine in Ehrlich ascites tumor cells.

Intact Ehrlich ascites tumor cells extensively catabolize guanosine with the concurrent release of free guanine into the incubation medium (11). The cleavage of guanosine

TABLE IX

A time study of adenosine metabolism by mouse erythrocytes in vitro

Incubation period (minutes)	Concentrations (mM)		
	Adenosine	Inosine	Hypoxanthine
0	2.03	0.02	0.01
5	1.68	0.30	0.10
15	0.97	0.88	0.29
30	0.17	0.42	0.49

NOTE: Mouse erythrocytes (0.1 ml packed cells per ml incubation mixture) were incubated with adenosine-8-^{14}C (2.1 mM; specific activity, 3.2 x 10^5 c.p.m. per μmole) under the usual experimental conditions for the periods indicated in the table. Incubation mixtures (complete) were extracted with perchloric acid, and analyzed as described in Table I.

Red blood cells were prepared as follows: Mice were injected intraperitoneally with 0.1 ml heparin (10 mg per ml). After 10 minutes animals were anesthetized with ether, and blood collected. The red cells were then washed 3 times with isotonic saline and packed by centrifugation at 2,400 x g for 10 minutes.

by a purified fraction from Ehrlich ascites tumor cells has also been described (12). In the present studies, the metabolic fate of guanosine in cells of the Ehrlich ascites tumor *in vitro* has been investigated in some detail.

Guanosine-8-^{14}C was incubated with tumor cells, and as described in Figure 4, the entire incubation mixture (cells plus medium) was analyzed for radioactive metabolites by paper electrophoresis. The electrophoretogram shown in Figure 4 indicates that at least four substances were derived from guanosine-8-^{14}C in the tumor cell system. Four of the five radioactive areas coincided with the carrier spots of guanine, xanthine, guanosine and GMP. An unidentified radioactive area (peak IV) was found between guanosine and GMP, as may be seen in this figure.

It was noted that the position of xanthosine and GMP overlapped in the above electrophoretic system. To determine the contribution that each made to the observed radioactivity in the GMP region, samples of the reaction mixture were subjected to two dimensional paper chromatography. The chromatogram was run first in solvent A and then in solvent D for the second dimension. Xanthosine is separated from GMP and other metabolites in this system. It was found that only a trace of radioactivity (less than 1% of the total) accompanied the xanthosine carrier on these chromatograms, indicating that xanthosine was only a minor

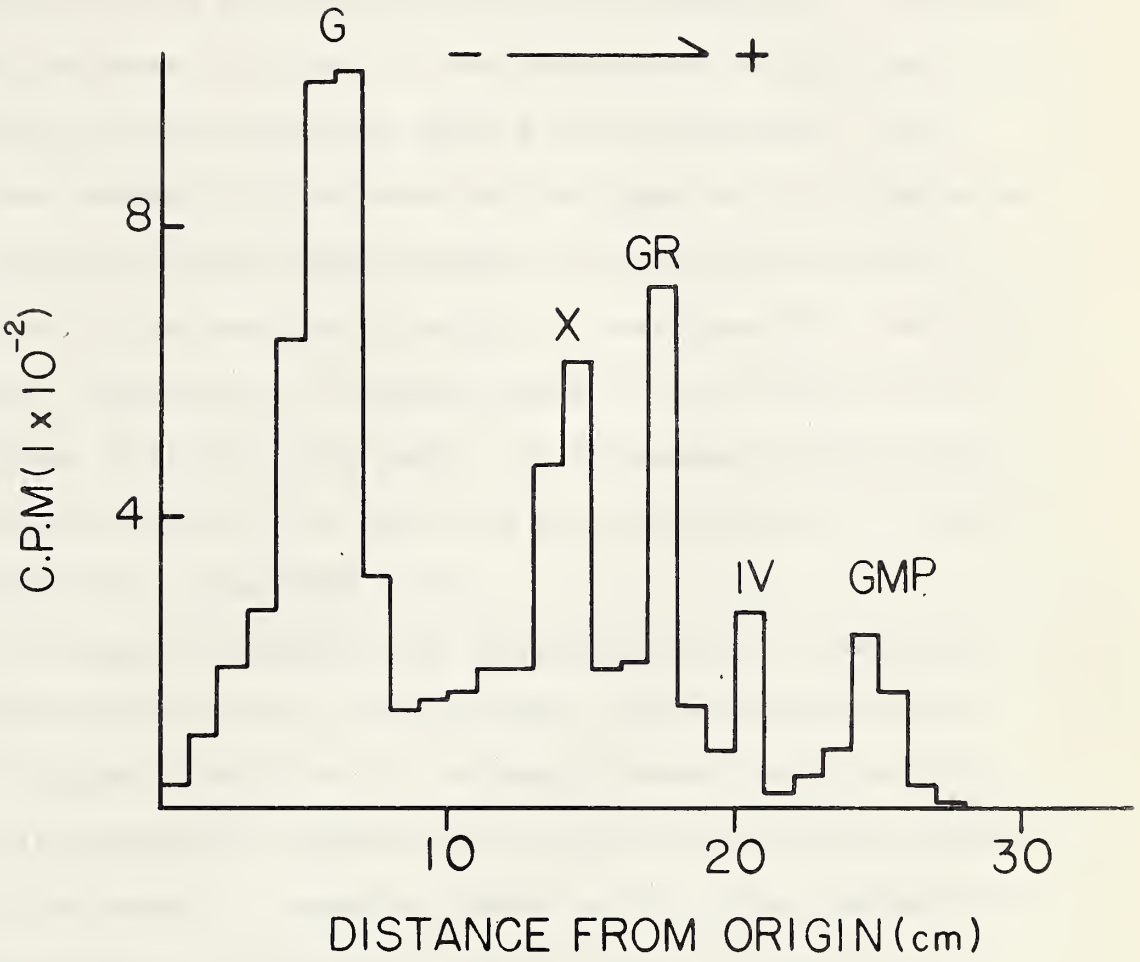

FIG. 4. Metabolic fate of guanosine in Ehrlich ascites tumor cells in vitro.

Tumor cells were incubated under usual conditions with guanosine-8-^{14}C (1 m$\underline{M}$; specific activity, 4.78 x 10^5 c.p.m. per μmole) for 30 minutes. The incubation mixture (complete) was extracted with perchloric acid and a 0.015 ml sample was subjected to electrophoresis with carriers. The radioactive peaks on the paper were identified as described in Figure 2.

product in the reaction mixture.

The radioactive peak (IV) which appeared between guanosine and GMP on the electrophoretograms was not an artifact, as it was found in several experiments. Further, as may be seen in Figure 5, the magnitude of this peak increased as the incubation period was lengthened. This unknown product did not move at the same rate as adenosine or inosine on electrophoretogram. Since xanthine was present in the reaction mixture, it was possible that the unknown represented a further stage in the catabolism of xanthine, possibly uric acid. In subsequent experiments, it was found that uric acid and the radioactivity of peak IV migrated at the same rate.

Attempts to confirm the identification of the peak IV metabolite as uric acid by paper chromatography were not successful because the solvent systems employed did not satisfactorily resolve the mixture of reaction products. For this reason, a reaction mixture with fewer components was employed to facilitate the isolation of uric acid. Guanine-8-^{14}C was used as the radioactive substrate since it appeared to be part of the metabolic sequence leading from guanosine to uric acid:

guanosine→guanine→xanthine→uric acid.

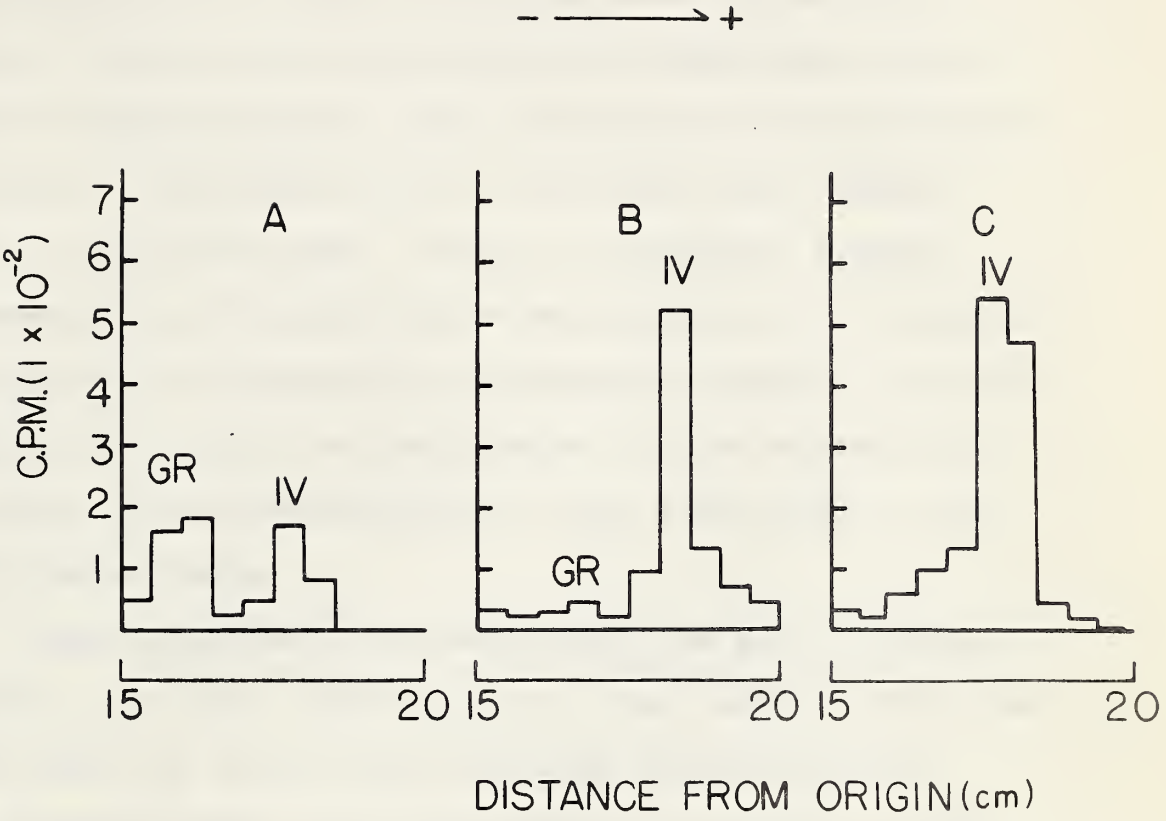

FIG. 5. The progressive increase in the magnitute of peak IV (uric acid) during the metabolism of guanosine by Ehrlich ascites tumor cells.

Tumor cells were incubated under the usual conditions with guanosine-8-^{14}C (0.11 m$\underline{M}$; specific activity, 4.25 x 10^6 c.p.m. per μmole) for the period indicated below. The medium fractions of these incubation mixtures were analyzed by electrophoresis.

Incubation period: A. 5 minutes
B. 15 minutes
C. 30 minutes

In the experiment shown in Figure 6, guanine-8-^{14}C was incubated with ascites tumor cells and only the medium fraction of the incubation mixture was analyzed by electrophoresis in order to further simplify analysis. Figure 6 shows that three radioactive areas were found on the electrophoretogram; these coincided with carrier spots of guanine, xanthine and uric acid which were visible under ultraviolet light. Figure 7 represents a paper chromatogram of the same reaction mixture run in solvent F. In this system radioactivity accompanied guanine, xanthine and uric acid, and the distribution of the radioactivity in these areas corresponded well with that found on the electrophoretogram.

These experiments indicated that the peak IV metabolite was uric acid. However, since this identification has been made only on the basis of rates of migration in the chromatographic and electrophoretic systems, it must remain tentative until confirmed by other properties such as the ultraviolet absorption spectrum, reaction with ammonical silver nitrate (62), and the ability to serve as a substrate for uricase.

2. Rate of guanosine utilization.

In the experiment of Figure 8, the rates of guanosine disappearance from the medium at different initial concentrations were measured. Because the concentration of cells in this experiment was 0.05 g per ml, intracellular

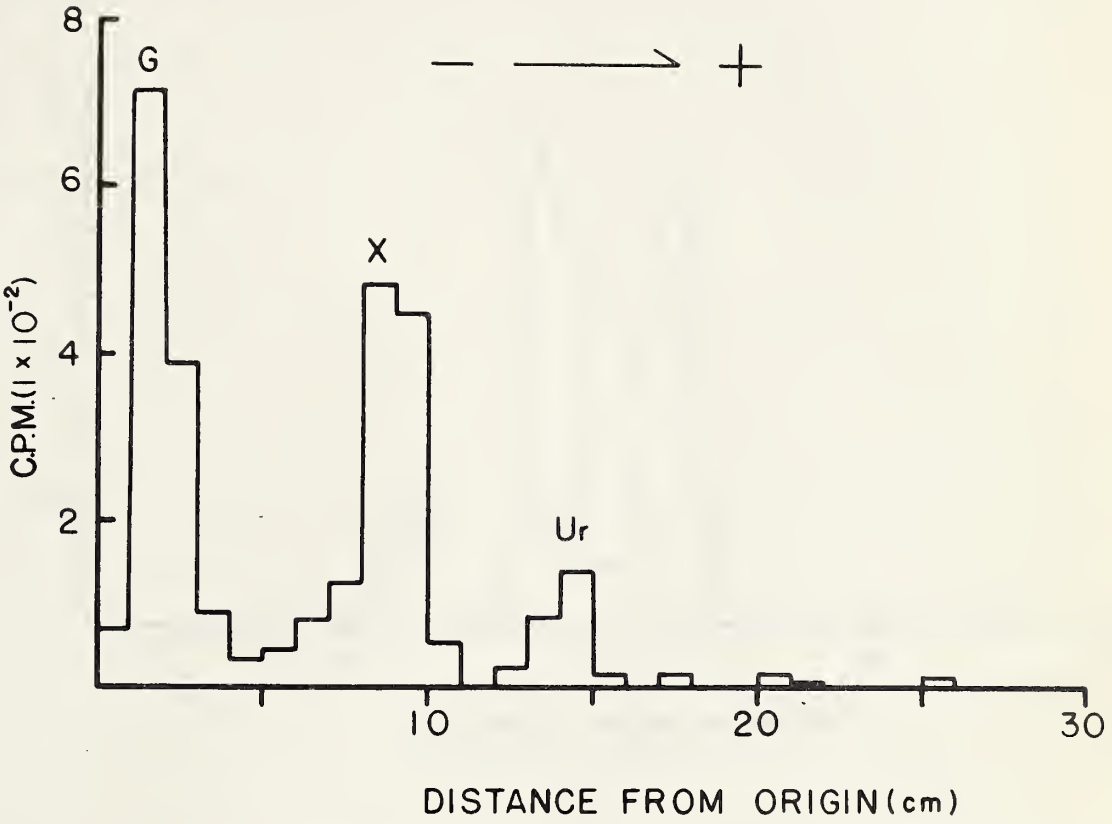

FIG. 6. Electrophoretogram of the medium fraction of an incubation mixture containing guanine-8-^{14}C and Ehrlich ascites tumor cells.

Tumor cells were incubated with guanine-8-^{14}C (0.084 m$\underline{M}$; specific activity, 4.03 x 10^6c.p.m. per μmole) under the usual conditions; a 0.01 ml sample of the incubation medium was analyzed by electrophoresis.

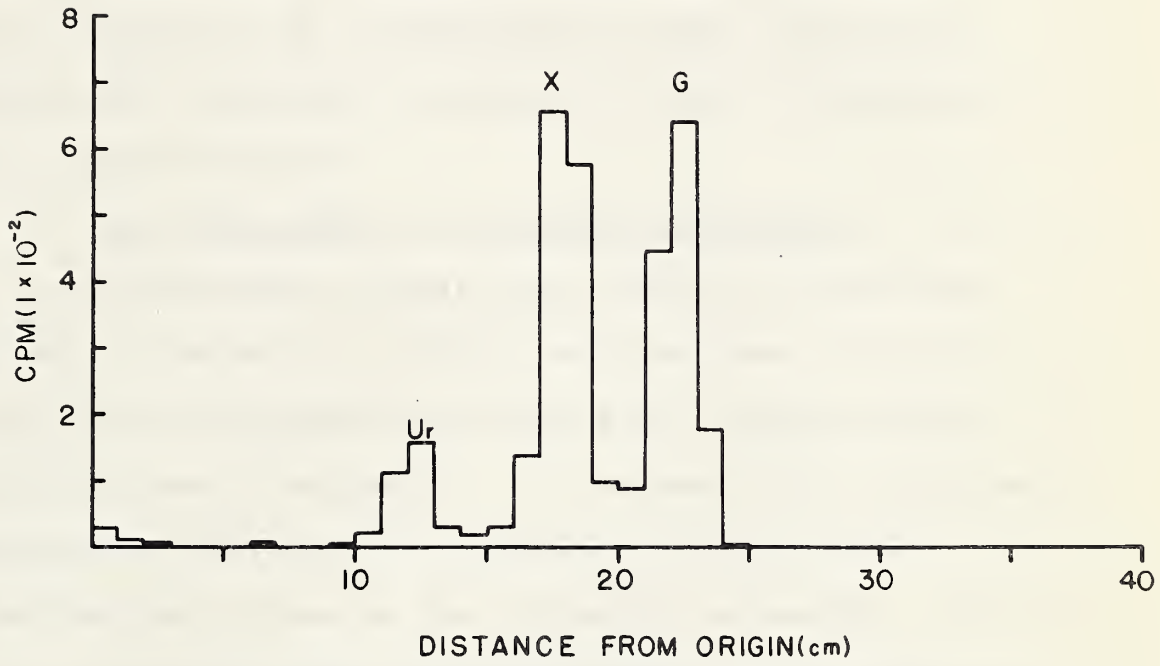

FIG. 7. Paper chromatogram of the medium fraction of an incubation mixture containing guanine-8-^{14}C and Ehrlich ascites tumor cells.

A 0.01 ml sample of medium from the experiment of Figure 6 was analyzed by paper chromatography in solvent F. Peaks of radioactivity were identified by their correspondence with marker compounds.

water approximated only 1.5% of the volume of reaction mixture (see "Materials and Methods"). Thus, any difference between the rate at which guanosine was actually metabolized and that at which it disappeared from the medium would be small. Figure 8 shows that when the initial concentration of guanosine was 1mM, its rate of disappearance approached a maximum at about 18 μmoles per gram of cells per hour.

3. The time course of guanosine metabolism.

In the experiment summarized in Table X, guanosine-8-^{14}C was incubated with Ehrlich ascites tumor cells and the incubation mixtures were sampled for analysis after various periods of incubation. It is seen that a progressive conversion of substrate into guanine, xanthine, and nucleotides took place as the incubation proceeded. Guanine was the principal catabolite of guanosine. The formation of uric acid was detected only in the later stages of the incubation. The rate of guanosine disappearance during the first 15 minutes of incubation was 16.4 μmoles per gram of cells per hour.

4. The synthesis of guanosine.

Ascites tumor cells and erythrocytes synthesize ribonucleosides by the transfer of ribose from a "donor" ribonucleoside to purine and pyrimidine bases (18,58). In Section A, inosine synthesis by tumor cells was discussed and it was shown that guanosine served as a ribosyl donor.

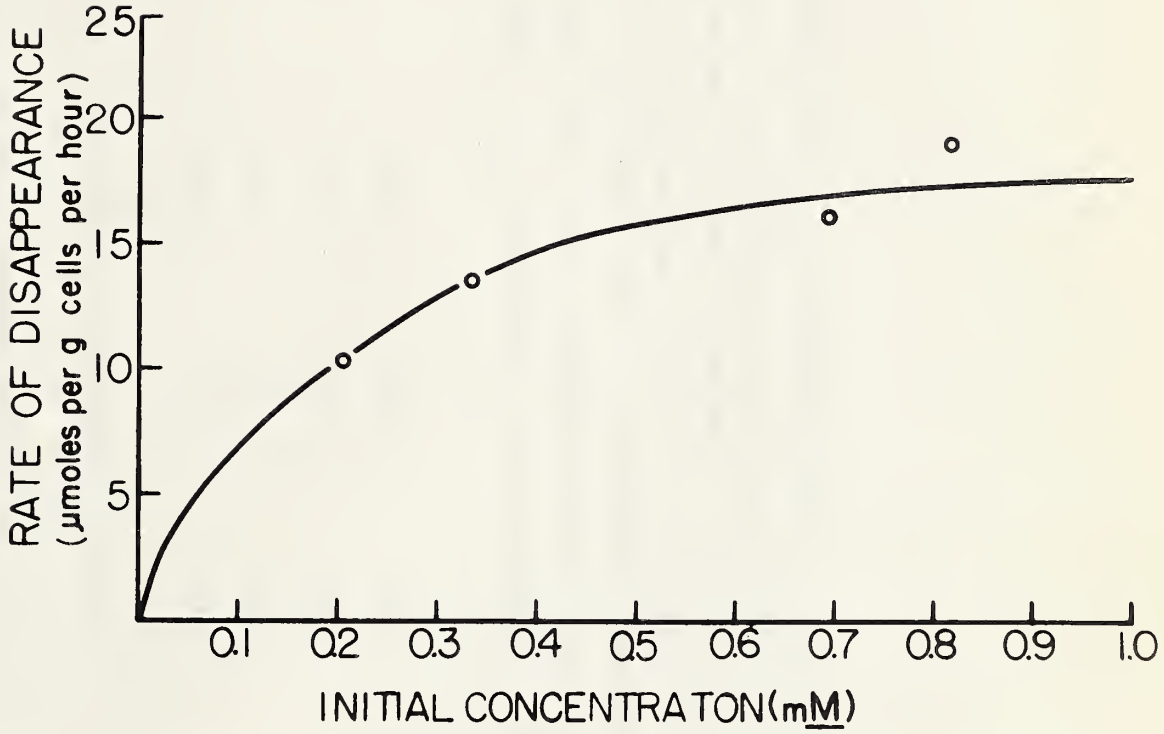

FIG. 8. Effect of concentration on the rate of guanosine utilization by Ehrlich ascites tumor cells.

Experimental conditions and analytical procedures were the same as described in Figure 3 except that the concentration of cells was 0.05 g per ml incubation mixture.

TABLE X

Time course of guanosine metabolism by Ehrlich ascites tumor cells in vitro

Time (Minutes)	Concentration of radioactive compounds in whole incubation mixture (mM x 10)				
	Guanosine	Guanine	Xanthine	Uric acid	Nucleotide
0	9.73	0.28	0.00	0.00	0.00
5	7.60	1.78	0.32	0.00	0.35
15	5.63	2.35	0.50	0.13	0.62
30	3.35	4.43	0.95	0.26	0.96

NOTE: Tumor cells were incubated with guanosine-8-^{14}C (1 mM, specific activity, 5 x 10^5 c.p.m. per μmole) for the indicated period under the usual conditions. Duplicate incubation mixtures were prepared for each time point. Incubations were terminated by the addition of perchloric acid and samples of the resulting extract were analyzed by paper electrophoresis. Concentrations of guanosine and its metabolites were calculated from the distribution of radioactivity on the electrophoretograms and the specific activity of the guanosine-8-^{14}C substrate.

The present experiments demonstrate the synthesis of guanosine by ascites tumor cells.

Guanine-8-^{14}C and various ribonucleosides were incubated together with intact Ehrlich ascites tumor cells and the medium fraction of the incubation mixture was analyzed by paper chromatography using solvent A. This solvent does not separate guanosine and xanthosine although both are well separated from their bases. However, since is was demonstrated by two dimensional chromatography (first dimension, solvent A, second dimension, solvent D) that no appreciable amount of xanthosine was formed in the incubation mixture, solvent A was judged to be adequate for the present purpose.

Measurements of guanosine synthesis in this system are hindered by the low solubility of guanine. Hence, the rates achieved were markedly lower than the rate of inosine synthesis. As can be seen in Table XI, inosine and uridine supported guanosine synthesis, whereas cytidine, adenosine were much less active in this respect. Xanthosine, orotidine, 4-amino-5-imidazolecarboxamide ribonucleoside, and Me-6MPR did not support guanosine synthesis.

In Table XI it may be noted that when guanine and guanosine were incubated together in the presence of the tumor cells, radioactivity was incorporated into guanosine. This probably represents an exchange reaction and not the net synthesis

TABLE XI

The synthesis of extracellular guanosine by Ehrlich ascites tumor cells in vitro

	Guanosine concentration in the medium (mM, x 10^2)		
	Expt. A	Expt. B	Expt. C
None	0.00	0.03	0.04
Adenosine	0.78	0.30	0.63
Inosine	2.52	2.47	1.82
Guanosine	2.08*	2.15*	
Xanthosine	0.00	0.03	
Me-6MPR			0.05
Cytidine	0.12	0.13	0.10
Uridine		1.74	
Orotidine		0.03	
4-amino-5-imidazole-carboxamide ribonucleoside		0.04	

NOTE: Tumor cells were incubated under the usual conditions with guanine-8-^{14}C (Expt. A and B, 0.084 mM; Expt. C, 0.093 mM) and various ribonucleosides (2mM). Duplicate incubation mixtures were prepared to test each variable. Samples of medium from each incubation mixture were chromatographed in solvent A with carrier guanosine. The radioactivity accompanying the carrier and the initial specific activity of guanine-8-^{14}C were used to calculate the amount of guanosine synthesized by the tumor cells.

* When guanine-8-^{14}C and guanosine are incubated with tumor cells, an exchange takes place yielding guanosine-8-^{14}C. Thus, the data presented do not represent the net synthesis of guanosine, but the amount of guanine-8-^{14}C which has been converted to guanosine. As explained in the text, the method of calculation underestimates the extent of this conversion.

of guanosine, the catabolism of which probably continues under these reaction conditions. Similar exchange reactions for uracil-2-^{14}C and uridine and for hypoxanthine-8-^{14}C and inosine have been reported from this laboratory (18). The values listed undoubtedly underestimate the amount of guanine-8-^{14}C converted to guanosine by the exchange reaction because they were calculated using the initial specific activity of the guanine-8-^{14}C substrate. Since dilution of the guanine radioactivity would occur as a consequence of the exchange reaction with non-isotopic guanosine and as a consequence of cleavage of the latter (18), the actual amount of guanine incorporated into guanosine must be higher than the data of Table XI would indicate. Thus, this experiment indicated that Ehrlich ascites tumor cells synthesize guanosine and catalyze an exchange reaction between guanine and guanosine with the products of both processes appearing in the incubation medium.

The appearance in the incubation medium of a ribonucleoside product which can be readily catabolized is an unusual feature of these observations. It has been reported that Ehrlich ascites tumor cells release purine ribonucleoside phosphorylase into the supporting medium during incubation in vitro (18). The experiments reported in Table XII were intended to determine whether the observed synthesis of guanosine was catalyzed by enzymes

in the medium. Postincubation medium was prepared as described in an earlier section, that is, by incubating tumor cells under the usual conditions but without substrate. After removal of the cells by centrifugation, substrates were added to the cell-free medium and the incubation was repeated. It is apparent from the data in Table XII that enzymes present in the medium accounted for only a part of the synthesis of extracellular guanosine. Thus, guanosine synthesis and the guanine-guanosine exchange reaction are properties of the tumor cells.

The effects of several compounds on guanosine synthesis were also studied. It is seen in Table XIII that guanosine synthesis was increased by 60% in the presence of iodoacetate or arsenate, and by 80% if 2,4-dinitrophenol was added to the mixture; Me-6MPR depressed this reaction by 60%.

It has been demonstrated in this laboratory that Me-6MPR inhibits various aspects of ribonucleoside metabolism by intact cells and that these inhibitions are not manifested in broken cell preparations (57). The experiments of Table XIV surveyed the effects of this analogue ribonucleoside on guanosine metabolism. It can be seen in this table that Me-6MPR inhibited guanosine cleavage, exchange, and synthetic reactions and, as well, the formation of nucleotide from guanosine. Me-6MPR did

TABLE XII

Is the synthesis of extracellular guanosine catalyzed by enzymes released into the medium by Ehrlich ascites tumor cells during incubation?

Ribosyl donor	Guanosine synthesis in postincubation medium as a percentage of that in the presence of Ehrlich ascites cells	
	Expt. A	Expt. B
Uridine		43
Inosine	25	23
Guanosine	10	11

NOTE: Experimental conditions and analytical procedure were same as described in Table XI. Postincubation medium was prepared as described in Table II.

TABLE XIII

Enhancement and inhibition of the synthesis of extracellular guanosine by Ehrlich ascites tumor cells in vitro.

	Guanosine synthesized (% of control)	
Additions	Expt. A	Expt. B
Control	100	100
Potassium iodoacetate	143	156
Sodium arsenate		160
2,4-dinitrophenol		180
Me-6MPR	42	32

NOTE: As described in Table XI, tumor cells were incubated with guanine-8-^{14}C (Expt. A, 0.084 m$\underline{M}$; Expt. B, 0.114 m$\underline{M}$) and inosine (2m$\underline{M}$) and the amount of guanosine appearing in the incubation medium was measured. The concentration of each additive was 2m$\underline{M}$.

TABLE XIV

Requirement of cell integrity for the inhibition of guanosine metabolism by Me-6MPR.

Substrates*	Products	Product formed in the presence of Me-6MPR as a percentage of that in its absence.	
		Intact cells	Broken cells
GR-8-^{14}C	bases**	45	99
GR-8-^{14}C	nucleotides+	56	100
G-8-^{14}C + HR	GR-8-^{14}C	42++	102
G-8-^{14}C + GR	GR-8-^{14}C	17++	99

NOTE: Tumor cells (broken or intact) were incubated under the usual conditions with the substrates listed above in the presence or absence of Me-6MPR (2m$\underline{M}$). Unless otherwise specified, perchloric acid extract of whole incubation mixtures were analyzed. Paper chromatography in solvent A was used to isolate reaction products.

*Substrate specifications were as follows: GR-8-^{14}C, 2m$\underline{M}$ (specific radioactivity, 4.19 x 10^5 c.p.m. per μmole); G-8-^{14}C, 0.11 m$\underline{M}$ (specific radioactivity, 9.9 x 10^5 c.p.m. per μmole), non-radioactive inosine or guanosine, 2m$\underline{M}$.

** Includes the cleavage product, guanine, and its catabolites, xanthine and uric acid.

+ Compounds found in the nucleotide area of the paper chromatograms.

++ Medium only was analyzed.

not inhibit these reactions in broken cell preparations.

The synthesis of guanosine by mouse erythrocytes was examined in the experiments of Table XV, again because erythrocytes are frequently present in preparations of ascites cells. These data demonstrate that mouse erythrocytes catalyzed an exchange between guanine and guanosine which took place at a rate about 80% of that found for ascites tumor cells (Table XI). Inosine supported the synthesis of guanosine by erythrocytes at a rate which was about 10% of that observed in a similar experiment with tumor cells (Table XI). Uridine did not support guanosine synthesis by erythrocytes, in agreement with other observations on the inability of mouse erythrocytes to metabolize uridine (18).

TABLE XV

Synthesis of extracellular guanosine by mouse erythrocytes

Ribosyl donors (2mM)	Guanosine concentration (mM, x 10^2)	
	Expt. A	Expt. B
None	0.13	0.04
Inosine	0.24	0.17
Guanosine	1.41	1.69
Uridine		0.03

NOTE: Incubation mixtures contained guanine-8-^{14}C (0.093 mM; specific activity, 1.15 x 10^6 c.p.m. per μmole), ribonucleosides and red blood cells (0.1 ml packed cells per ml incubation misture). After incubation, the cells were removed by centrifugation and supernatant was heat killed, clarified, and then analyzed by paper chromatography in solvent A. Red blood cells were prepared as described in Table IX.

IV DISCUSSION

A). Adenosine Metabolism

The present studies have demonstrated clearly that adenosine is deaminated by intact Ehrlich ascites tumor cells and the deamination product, inosine, is further metabolized to yield the free base, hypoxanthine. Direct cleavage of adenosine by the tumor cells probably does not occur, because (a) adenine was not found as a metabolite of adenosine-8-^{14}C and (b) adenosine did not serve as a ribosyl donor in the synthesis of ribonucleosides. Paterson (18) has shown that adenine also does not serve as a ribosyl acceptor in this tumor cell system, a further indication that adenosine phosphorylase is absent.

An unusual feature of adenosine metabolism in this system was that inosine appeared in the extracellular medium. It was found that inosine, when provided in the medium, was cleaved by these cells at least three times more rapidly than adenosine was deaminated; however, in spite of these circumstances, inosine was released into the medium during the metabolism of adenosine. At the most, deamination of adenosine by enzymes in the extracellular medium accounted for about 40% of the formation of extracellular inosine. These data might lead one to speculate that deamination could occur on the cell surface, since surface-located enzymes have been recognized in several cell types (63,64,65,66).

However, this possibility is weakened by the fact that the adenosine deaminase of Ehrlich ascites tumor cells is a soluble enzyme (10). Furthermore, time studies of adenosine metabolism with the tumor cells indicated that inosine accumulated inside the cells and then moved outward. Thus, it appears that adenosine deamination takes place inside the tumor cell. Ribonucleoside phosphorylases are also soluble enzymes (11,18,42) and likewise, ribonucleoside cleavage probably occurs within the tumor cell. These considerations, together with the fact that these cells utilize inosine at least three times more rapidly than they are able to deaminate adenosine, make the observed appearance of extracellular inosine during adenosine metabolism seem rather puzzling. An explanation of this anomaly may be found in biochemical "compartmentation" within the cell.

Biochemical compartmentation is adduced when substrate and enzyme are known to be present within the cell, but do not react; when metabolic pathways have an identical product, and yet product from one can be differentiated from that of the other; when the metabolism of an intracellularly synthesized compound is different from that of the same compound supplied from without. The confinement of hydrolases in lysosomes and the localization of enzymes of tricarboxylic acid cycle in mitochondria are well known examples of compartmentation which have morphological bases (67). In Ehrlich ascites tumor cells,

some aspects of the metabolism of purine compounds can be described in terms of intracellular compartmentation that has no apparent morphological basis. Paterson and Hori (10) reported that 6MPR was converted to 6MP ribonucleotide by Ehrlich ascites tumor cells, whereas this conversion did not occur in intact cells of a 6MP-resistant subline. Since both cell types have an equal capacity for cleavage of 6MPR (11), and both have equal intracellular concentrations of inosinate pyrophosphorylase (68), the enzyme responsible for 6MP nucleotide synthesis, they speculated that 6MPR was cleaved in resistant cells at a site where the purine product of the cleavage reaction was not available for inosinate pyrophosphorylase (11). Kandaswamy and Henderson (69) showed that ethidium bromide inhibited completely the incorporation of purine bases into nucleic acid, but only moderately inhibited that of glycine. They also demonstrated that the conversion of bases into nucleotides was not inhibited by this drug. These authors interpreted their result to mean that nucleotides formed by de novo synthesis and from exogenous bases entered into a common pool from which they were incorporated into nucleic acid; ethidium bromide was thought to block entry of the latter nucleotides into this pool. This interpretation implies that

de novo synthesis of nucleotide and the formation of nucleotide from preformed bases occur in two different cell compartments. Likewise, the appearance of inosine in the extracellular medium during the metabolism of adenosine by the tumor cells might also be due to some sort of intracellular compartmentation in which adenosine was deaminated at one site and inosine cleaved at another. The inability of adenosine to support inosine and uridine synthesis in spite of the fact that its deamination prodcut, inosine, is an effective supporter of these reactions, may be another aspect of compartmentation. Paterson (18) also demonstrated that cytidine did not support synthesis of extracellular inosine and uridine by Ehrlich ascites tumor cells, even though the deamination product, uridine, was very effective in this regard.

The 6MP-resistant subline of Ehrlich ascites tumor developed by Paterson (18) has been shown to differ in biochemical properties from the parent line in being unable to synthesize 6MP ribonucleoside-5'-phosphate and in the deletion of a nuclear 5'-nucleotidase (39). In the present studies it was found that cells of these two tumor lines differ in their ability to utilize adenosine, cells of resistant subline being more active in this respect. Since the two cell lines do not appear to differ in ribonucleoside cleavage, synthesis, and exchange reaction[7] it appears that the above difference might be due to rate differences in the deamination or possibly in the phorphorylation of adenosine.

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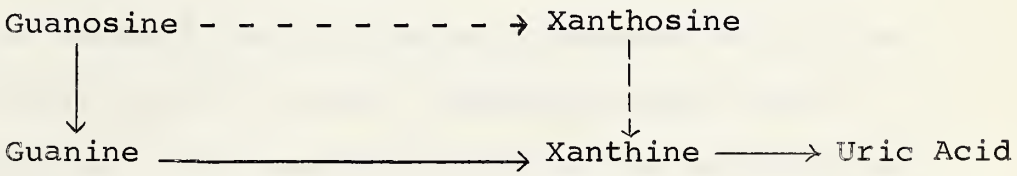
7. A.R.P. Paterson and A.I. Simpson, unpublished results.

B). Guanosine Metabolism

Guanosine was rapidly cleaved to guanine by these tumor cells. Xanthine and uric acid were recognized as products of the further catabolism of guanine, and some incorporation into nucleotide was observed. No significant amount of xanthosine was formed during the incubation of tumor cells with guanosine. The xanthine formed in the incubation mixture, probably was not formed by way of xanthosine because (a) xanthosine was not demonstrable, (b) xanthosine is catabolized at a low rate by these cells when intact (11), and, (c) xanthosine is cleaved at a lower rate than other natural purine ribonucleosides by purified purine ribonucleoside phosphorylase (70,71).



The presence of radioactive uric acid, derived from guanosine, in the incubation mixture was demonstrated by electrophoretic means. In addition, it was shown that guanine was degraded to both xanthine and uric acid by the tumor cells, indicating that xanthine was the immediate precursor of uric acid. As xanthine oxidase has been reported to be absent from Ehrlich ascites tumor cells (72), the presence of uric acid was unexpected.

However, the rate of uric acid formation was low; only 2-4% of the radioactivity from guanosine-8-^{14}C was recovered as uric acid, whereas about 70% of the guanosine was metabolized after an incubation period of 30 minutes. Moore and LePage (33) have shown that 6-thioguanine was converted to 6-thioxanthine and 6-thiouric acid by Ehrlich ascites tumor cells. It is possible that the oxidation of xanthine may have been caused by cells other than tumor cells present in the preparation such as leucocytes.

The conversion of guanosine to nucleotide was demonstrated in these experiments and would be expected to take place by the action of inosinate-guanylate pyrophosphorylase, which is present in these tumor cells (10,37,68). It has been demonstrated that ribonucleosides will support the synthesis of nucleotides by the ascites tumor cells (36,73). Ribonucleosides, upon phosphorolytic cleavage, contribute to the intracellular pool of ribose phosphates and thence to PRPP, which participates in nucleotide synthesis by the pyrophosphorylase route (30). The existence of kinases that phosphorylate inosine and guanosine has been questioned (74,75).

When guanine-8-^{14}C was incubated with appropriate "donor" ribonucleosides, guanosine-8-^{14}C was synthesized. An exchange of isotope between guanine-8-^{14}C and guanosine

was also demonstrated. It was observed that some ribonucleosides which were readily cleaved by the tumor cells, such as uridine, guanosine, and inosine were effective supporters of these reactions, while adenosine and cytidine, which are not directly cleaved, were much less effective in this respect. Xanthosine, which is cleaved by these cells at a low rate, did not support guanosine synthesis. These data are generally consistent with the idea that the synthesis of guanosine was catalyzed by nucleoside phosphorylases. However, this simple explanation does not account for some features of this reaction such as the enhancement in guanosine synthesis by iodoacetate and arsenate. It has been reported that arsenate inhibited the transribosylation activity (ribonucleoside synthesis involving a base and a ribonucleoside which donates its ribosyl group) of partly purified bacterial preparations by competing with phosphate (60). It is well known that arsenate will take place of phosphate in the cleavage of nucleosides by phosphorylases; the arsenolytic reaction yields free ribose because of the spontaneous hydrolysis of the intermediate, ribose-1-arsenate (76). This fact has been taken as an indication of involvement of ribonucleoside phosphorylase in the bacterial ribose transfer reactions (60). Therefore, in the present work it might have been expected that arsenate would inhibit the synthesis of guanosine.

However, it was found that arsenate enhanced the reaction by 60%. Similar effects of arsenate on the synthesis of uridine and inosine and on the inosine exchange reaction have been reported by Paterson and Simpson (77), who suggest that arsenate may exert this effect by reducing the catabolism of the ribose phosphates. The effects of iodoacetate and 2,4-dinitrophenol on guanosine synthesis are puzzling. Iodoacetate enhanced the guanosine synthesis to the same extent as arsenate, in parallel with other observations from this laboratory on synthesis of inosine and uridine (77). It has been reported that iodoacetate strongly inhibits phosphoribomutase (78) and triose phosphate dehydrogenase (79,80). Inhibition of the above two enzymes would be expected to preserve ribonucleoside-derived supplies of ribose phosphates which might then support guanosine synthesis. However, iodoacetate is known to be a potent inhibitor of purified ribonucleoside phosphorylase (81,82). Thus, it is difficult to explain why this compound should promote guanosine synthesis if these synthetic reactions are, indeed, catalyzed by ribonucleoside phosphorylases. Similarly, 2,4-dinitrophenol inhibited phosphorolysis of uridine *in vitro* by Ehrlich ascites tumor cells and by purified uridine phosphorylase from this source (42), and also inhibited purine ribonucleoside phosphorylase

isolated from human erythrocytes (81). However, this compound unexpectedly promoted guanosine synthesis by 80% in these experiments. These puzzling results tend to support the idea that guanosine synthesis in this system might be mediated by enzymes other than phosphorylases. In any case, it is obviously difficult to apply facts learned from isolated and purified enzymes to the very complex situation found in the functioning, intact cell.

C) Effects of Me-6MPR on Adenosine and Guanosine Metabolism

These studies have shown that Me-6MPR inhibited adenosine metabolism by intact tumor cells, but had no effects on adenosine metabolism by broken cells or by postincubation medium. However, the inhibition was only moderate. On the contrary, this purine analogue ribonucleoside markedly inhibited guanosine metabolism. Nucleotide synthesis from guanosine, guanosine cleavage, the guanine-guanosine exchange reaction, the inosine-supported synthesis of guanosine, and the reverse reaction, the guanosine-supported synthesis of inosine, were all markedly inhibited by this compound. For these inhibitory effects to be manifested, the cells had to be whole. These findings parallel others made in this laboratory on the effects of Me-6MPR on uridine and inosine

metabolism, and are compatible with the hypothesis that Me-6MPR blocks the entrance of ribonucleosides into the cell. These characteristics of Me-6MPR inhibition also imply that an enzymatic system is responsible for the transport of nucleosides across the cell membrane. If this proves to be true, then the fact that adenosine metabolism was less affected by Me-6MPR than that of guanosine and other ribonucleosides might mean that the mechanism by which adenosine traverses the cell membrane is different from that for other ribonucleosides, the latter being more sensitive toward Me-6MPR than the former. It is also possible that adenosine might have a higher affinity for a common transport system than other ribonucleosides, and thereby its metabolism might be less affected by Me-6MPR than that of other nucleosides. However this possibility seems less likely than the first one, for it has been demonstrated in this laboratory that adenosine has a small effect on uridine-supported synthesis of inosine (57).

V. Summary

A. Adenosine Metabolism

During the metabolism of adenosine by Ehrlich ascites tumor cells in vitro, inosine appeared in the extracellular medium. This finding seemed unusual because inosine is known to be readily catabolized by these cells and was investigated further by testing a number of possible explanations.

One of these possibilities was that the cells might possess a higher activity for the deamination of adenosine than for phosphorolysis of inosine. However, the converse was found to be true; it was found that the tumor cells were able to cleave inosine at least 3 times faster than they could deaminate adenosine. The possibility of extracellular deamination of adenosine was considered next. The Ehrlich ascites cells in vitro released some adenosine deaminase into the incubation medium; however, it was found that, at the most, the extracellular enzyme accounted for about 40% of the formation of extracellular inosine. The possibility of cell surface deamination seemed unlikely because adenosine deaminase is a soluble enzyme. Moreover, it was found that during adenosine metabolism, concentrations of inosine were always higher inside the tumor cells than outside, suggesting that the deamination of adenosine was intracellular.

From these observations it was postulated that adenosine was deaminated in the tumor cell at a site or in a compartment which was different from that in which inosine was phosphorolyzed. Inosine thus formed, might not be directly available for phosphorolysis, and so might move outward. The inability of adenosine to support the synthesis of extracellular inosine and uridine affords further support to this idea.

The abilities of the Ehrlich ascites tumor and its 6MP-resistant subline to metabolize adenosine were compared. It was found that cells of resistant subline had higher ability to metabolize adenosine than cells of its parent line.

B). Guanosine Metabolism.

Guanosine was converted by the tumor cells in vitro to guanine, xanthine, uric acid, and nucleotide. Xanthosine was not found in the incubation mixtures, indicating that guanosine was not deaminated by these cells. Xanthine apparently arose by deamination of the guanosine cleavage product, guanine, and was further oxidized at a low rate to uric acid.

Extracellular guanosine was synthesized from guanine in the presence of appropriate "donor" ribonucleosides. Inosine and uridine supported this reaction, but adenosine and cytidine were much less effective in this respect. Xanthosine, 4-amino-5-imidazolecarboxamide ribonucleoside,

Me-6MPR, and orotidine were completely inert in these reactions. An exchange reaction between guanine-8-^{14}C and guanosine was also demonstrated. The synthesis of extracellular guanosine and guanine-guanosine exchange reaction were demonstrated to be properties of the tumor cells. Enzymes in the extracellular medium contributed only a fraction of the extracellular guanosine formed. Several compounds were found to affect these ribose transfer reactions. Arsenate, iodoacetate, and 2,4-dinitrophenol enhanced the inosine-supported synthesis of guanosine while Me-6MPR inhibited this reaction.

C). Effects of Me-6MPR on Adenosine and Guanosine Metabolism.

In agreement with other observations concerning the inhibitory effects of Me-6MPR on the metabolism of inosine and uridine, this compound was found to strongly inhibit synthetic, exchange and cleavage reactions with guanosine and, as well, to inhibit the conversion of guanosine into nucleotides. Adenosine metabolism was less affected by Me-6MPR, a finding which has some implications towards the mechanism of ribonucleoside transport across the cell membrane.

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